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13. ABSTRACT (Maximum 200 Words) We have hypothesized that the phytoestrogen, genistein, exerts its chemopreventive actions by postnatally programming developmental modifications to genes/proteins that render the mammary gland less susceptible to cancer. The objective of this proposed research is to identify regulatory proteins responsible for conferring breast cancer protection. The specific aims are 1) to identify proteins that are differentially expressed in mammary glands of rats treated \pm the carcinogen, DMBA, and the chemopreventive agent, genistein, and 2) to collect proteins from interstitial fluid surrounding mammary glands of rats, and to identify and characterize the major proteins that are modulated by DMBA and genistein. Using 2-D gels and mass spectrometry, we have determined that GTP-cyclohydrolase 1, a protein that plays a prominent role in the production of tetrahydrobiopterin (BH4), is modulated by genistein. BH4 is an essential co-factor for the enzyme tyrosine hydroxylase. The latter is demonstrated to be up regulated at 50 days, but not at 21 days, hence, we postulate that tyrosine hydrolase expression is regulated <i>via</i> a programming mechanism. Up regulated tyrosine hydroxylase is associated with increased production of dopamine, the latter has been reported to inhibit angiogenesis. Aim 2 has been initiated.				
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INTRODUCTION

Breast cancer is the most common malignancy diagnosed in American women. Even with improved technology for early detection and aggressive therapeutics, most often the disease is incurable once it is discovered. We believe that prevention rather than therapy is the desired future against cancer, and that innovative approaches and new technology will be the key to breakthroughs. Towards this, our laboratory has been studying how developmental alterations to the mammary gland can program against this cancer. More specifically, we have demonstrated that prepubertal only, and prepubertal plus adult, exposure(s) to dietary genistein, a phytoestrogen component of soy, confer(s) a long-term protective effect against dimethylbenz(a)anthracene (DMBA)-induced mammary cancer in rats (1). Consistent with our findings are epidemiological reports that Asians exposed to a diet high in soy during adolescence have a lower incidence of breast cancer (2, 3). We have hypothesized that genistein exerts its chemopreventive actions by postnatally programming developmental modifications to genes/proteins that render the mammary gland less susceptible to cancer. The objective of this proposed research is to identify regulatory proteins responsible for conferring breast cancer protection using innovative aims and technology. The specific aims are:

- 1) to identify proteins that are differentially expressed in mammary glands of rats treated \pm the carcinogen, DMBA, and the chemopreventive agent, genistein. (Projected for year 1).
- 2) to collect proteins from interstitial fluid surrounding mammary glands of rats, and to identify and characterize the major proteins that are modulated by DMBA and genistein. (Projected for years 2 and 3).

BODY

Specific Aim 1) To identify proteins that are differentially expressed in mammary glands of rats treated \pm the carcinogen, DMBA, and the chemopreventive agent, genistein.

Animal Treatments. We have used three genistein exposure protocols for investigating the proteome in mammary glands of female Sprague Dawley rats. Dietary exposure to genistein consisted of feeding 250 mg genistein/kg AIN-76A diet to the dam and offspring prepubertally while controls received AIN-76A diet only (Protocol A). From birth until approximately 14 days postpartum, the offspring are exposed only via nursing the mother's milk, thereafter the offspring also start eating the diet (4). Protocol B is life-time \pm genistein in the diet, plus DMBA on day 50 postpartum. Protocol C is the prepubertal injection model (500 ug genistein/g BW, subcutaneously on days 16, 18 and 20 postpartum) that has also been shown to protect against DMBA induced mammary cancer (5). Each group contained 10 rats.

2-D PAGE. Rats were killed and the fourth abdominal mammary glands were dissected, snap-frozen in liquid nitrogen and stored at -80C. Frozen tissues were pulverized and homogenized in 2-D lysis buffer. The samples were applied to the immobilized pH gradient (IPG) strips and allowed to rehydrate overnight. IPG strips were placed onto a Multiphor II electrophoresis unit (Amersham) and a current gradient applied for 24 hours (1-D). After the 1st dimension was complete, the strips were electrophoresised on a 1.0 mm 10-20% SDS PAGE gel (Criterion, Bio-Rad) on a vertical electrophoresis unit (2-D). The gels were fixed and stained with Sypro Ruby gel stain (Molecular Probes). Stained gels were scanned via a Perkin Elmer ProExpress densitometer and analyzed with the Progenesis (nonlinear) 2-D gel software system. Selected protein spots were prepared for MALDI-TOF analysis to get protein identification.

Protein Identification. Protein spots of interest were excised from the 2-D gels. These spots were destained and prepared for tryptic fragmentation. Tryptic digests of each protein were analyzed using a Voyager

MALDI TOF. Spectra produced were matched against a non-redundant protein database using MASCOT to determine the protein's identity.

RESULTS for Tasks a-c and f-h. For the initial study, proteomics was investigated in rats exposed via the diet (Protocol A to study imprinted proteins) and no significant difference in protein profiles were observed from mammary glands of 21 and 50 day old rats \pm genistein in the diet from birth until the end of the experiment. Next, we investigated the potential of pharmacologic doses of genistein via injection to provide a better opportunity to detect changes in protein profile (Protocol C). Figure 1, below, illustrates that 2-D gel electrophoresis resulted in the finding of a protein (spot 1307) being differentially regulated in 21 day old animals treated with genistein. Since the increase was significant ($p < 0.05$), this spot was picked for MALDI-TOF analysis. Studies of the tryptic peptide fragments identified the protein as guanosine triphosphate cyclohydrolase one (GTP-CH1). GTP-CH1 is the rate limiting enzyme in the production of tetrahydrobiopterin (BH4). This is an essential co-factor for the enzymes phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH) and the members of the nitric oxide synthase family (eNOS, iNOS and nNOS) (Figure 3 and Reference 6).

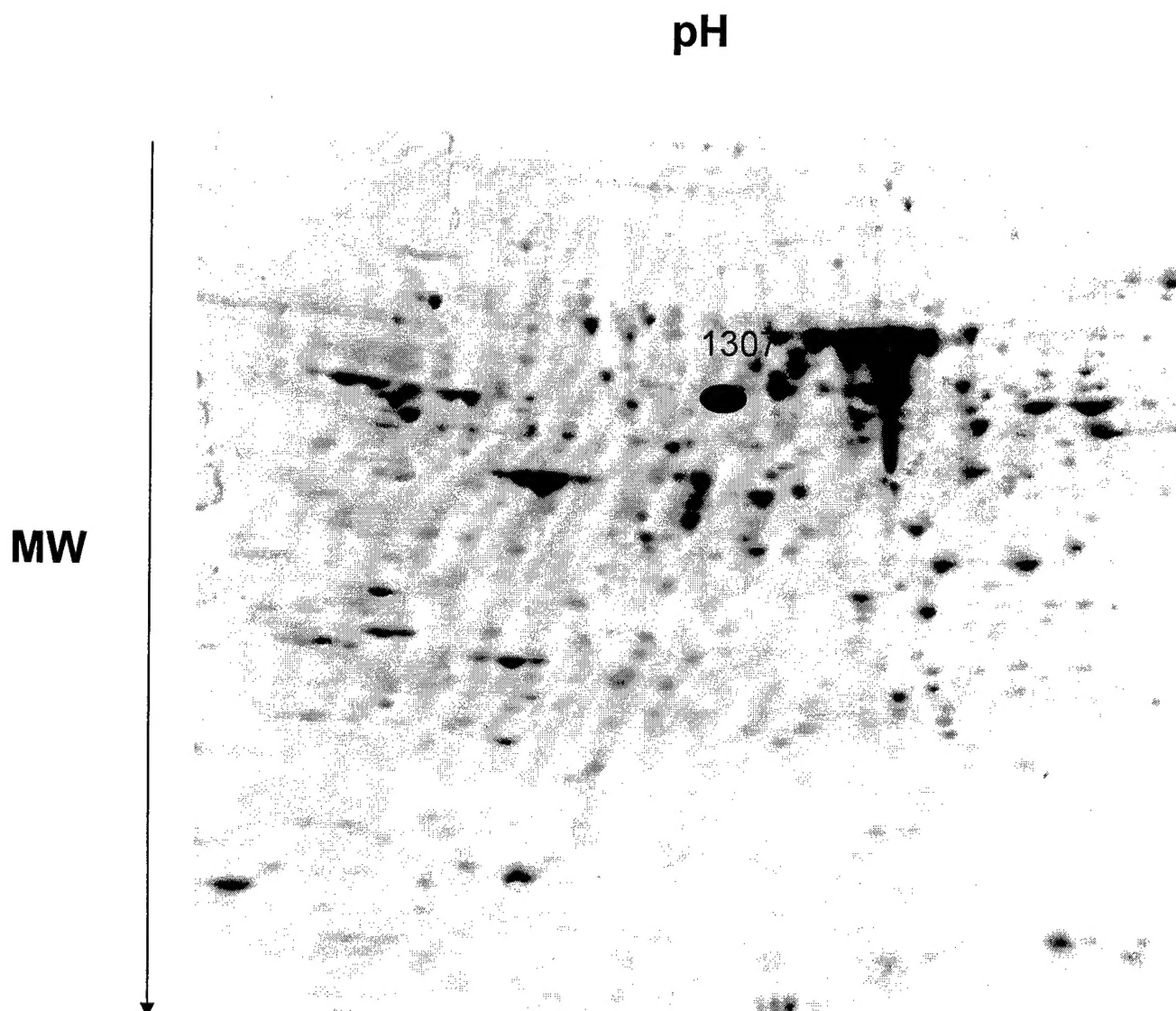


Figure 1. Representative 2-D gel of mammary gland of 21 day old rat.

Using a monoclonal antibody for GTP-CH1 (generously provided by Dr. Gregory Kapatos of Wayne State University), western blot analysis confirmed the increase in GTP-CH1 following genistein treatment at 21 days (Figure 2A). However, in mammary glands of 50 day old rats (30 days after the last genistein treatment) there was no difference between the treatment groups (Figure 2B).

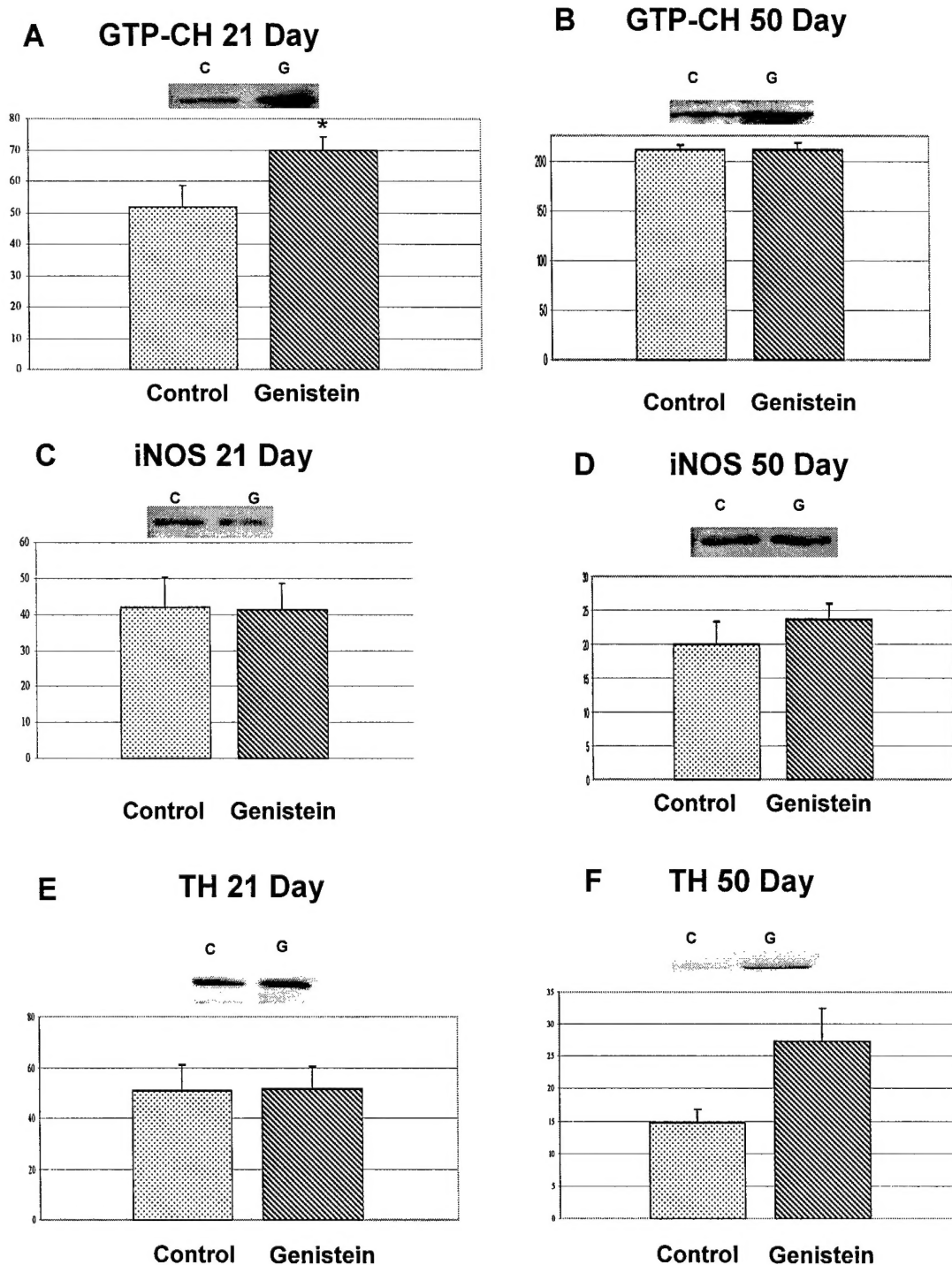


Figure 2. Western blot analysis for GTP-CH1 and downstream signaling molecules. Each group contained 8 samples). * $p < 0.05$ compared to age matched controls (Students t-test).

Having confirmed the change in GTP-CH1, we then investigated down-stream molecules that may be affected (Figure 3). We measured iNOS and TH expression and saw no significant change in expression for either protein in mammary glands of 21 day old rats (Figures 2C and 2E). However in 50 day old rats, we found that TH, but not iNOS, was significantly up-regulated in mammary glands of rats treated with genistein prepubertally only (Figure 2F and 2D).

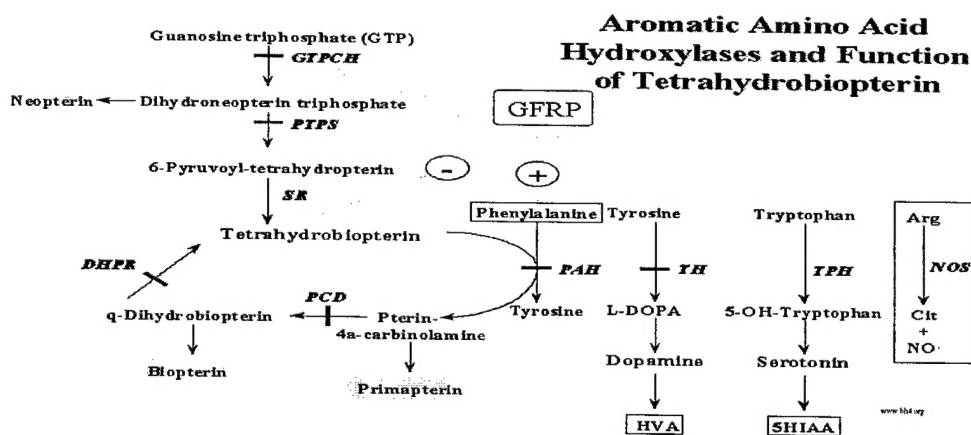


Figure 3. Graphical representation of proteins that are down-stream of GTP-CH. GTP-CH is the rate-limiting step in the production of tetrahydrobiopterin which is a necessary cofactor for the enzymatic activity of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase as well as the nitric oxide synthetases.

We have initiated the work on Tasks d and e of Aim 1. The animals were bred and the litters, including the offspring, were treated via Protocol B (life-time \pm genistein in the diet, plus DMBA on day 50 postpartum). At the time of this report, we have collected the mammary glands of 75 and 100 day old rats \pm genistein and \pm DMBA. One half of each mammary gland was immediately frozen for later proteomic analysis, and the other half was prepared for tissue pathology evaluation by our collaborator, Dr. Eltoun. In year 2, we will evaluate the proteome of these mammary glands relative to the treatments and pathology using 2-D gels/mass spectrometry.

Task i. The 2003 AACR meeting was attended and data from this project was presented.

Task j. Biostatistical and bioinformatic analyses of the data are in progress, and manuscript writing is pending confirmatory data.

Specific Aim 2. To collect proteins from interstitial fluid surrounding mammary glands of rats, and to identify and characterize the major proteins that is modulated by DMBA and genistein. (Projected for years 2 and 3). We have actually started this project. Figure 3 is a drawing of a microprobe that we have built for the purpose of collecting interstitial fluid from mammary glands of rats. We have actually implanted such probes in rats and are working to sustain and reproducibly extract fluid from the mammary glands. Then, it is our intent to analyze the proteins from the interstitial fluid by LC-MS/MS from rats treated \pm genistein and DMBA.

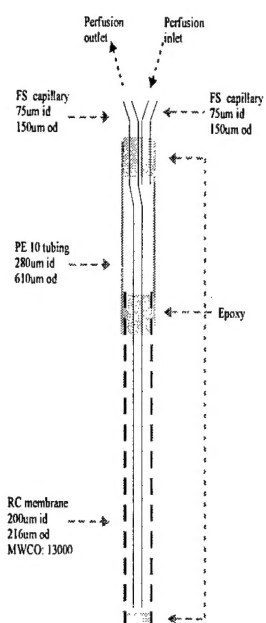


Figure 4. Schematic of microprobe for sampling of mammary interstitial fluid.

KEY RESEARCH ACCOMPLISHMENTS

Until recently, we were able to only look at known proteins that were suspected of being modulated based on their roles in other systems. Now however, we can use proteomic technologies to discover new proteins and/or identify new roles for known proteins. In the current study we discovered that the protein GTP-CH1 expression is significantly increased shortly following exposure to genistein. However, this was not a permanent effect because by day 50, in the absence of genistein, there was no difference between the treatment groups. By evaluating related metabolic pathways, we have been able to identify down-stream targets (5) and evaluate changes in these proteins in response to the changes of GTP-CH1. In the 21 day old animals we found no significant short-term changes in the TH and iNOS protein expression. However, at the 50 day time point there was significant up-regulation of TH expression. Since this difference is measurable 30 days after the final genistein treatment we postulate that some underlying programming effect on protein expression is manifested in the long-term expression profile of this downstream target.

Further investigations are warranted to understand how regulation of these proteins is occurring. Also, given that the primary role for TH is the production of dopamine (a compound associated with neuronal signaling) we must try to further understand the mechanisms by which the actions of TH are altering mammary gland susceptibility to carcinogenesis (6).

REPORTABLE OUTCOMES

C. Rowell, G. Puckett, K. Roarty, M. Kirk, L. Wilson, M. Carpenter and C. A. Lamartiniere, "Serum profiling and biomarker discover of rat mammary tumors using mass-coded abundance tags (MCAT)" In Proceedings of the 95th Annual meeting of the American Association for Cancer Research, **45**, 1203 Orlando, FL, 2004 .

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Carpenter, M., **Rowell**, C, Lamartiniere, C. and McCorkle, H., "2D-gel Proteomics in biomarker discovery." In Proceedings of Pharmaceutical Industry SAS Users Group 2004, San Diego, California (in press)

CONCLUSION

Using state-of-the-art proteomic technology, we have identified a protein that is differentially expressed following exposure to the phytoestrogen, genistein. This protein, GTP-cyclohydrolase 1, plays a prominent role in the production of tetrahydrobiopterin. The latter is an essential co-factor for the enzymes tyrosine hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase and the members of the nitric oxide synthase family (eNOS, iNOS and nNOS) (3). One of these enzymes, tyrosine hydroxylase, is up regulated at 50 days, but not at 21 days, hence we postulate that tyrosine hydroxylase expression is regulated *via* a programming mechanism. Up regulated tyrosine hydroxylase is associated with increased production of dopamine, the latter has been demonstrated to inhibit angiogenesis (7). Taken together, we postulate that prepubertal genistein exposure may be protecting against breast cancer susceptibility by increasing dopamine and inhibiting angiogenesis in mammary glands. Experiments are being designed to test this hypothesis.

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